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CANCER

Comm.
Dr. Andervont
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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
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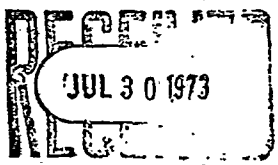
Application for Research Grant
(Use extra pages as needed)

Date: July 25,1973



1. Principal Investigator (give title and degrees):

Andrew Sivak, Ph.D.
Associate Professor of Environmental Medicine

2. Institution & address:

New York University Medical Center
550 First Avenue
New York, New York 10016

3. Department(s) where research will be done or collaboration provided:

Environmental Medicine

4. Short title of study:

Chemical and Viral Factors in the Etiology of Neoplastic Transformation

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: 3 Years

7. Brief description of specific research aims:

7.a. To examine in mammalian cell culture systems the possible cooperative and synergistic effects of sequential exposure to two different classes of carcinogens by:

7.a.1. Measuring the effects of prior treatment of mammalian cells with chemical carcinogens, cocarcinogens or mutagens on subsequent neoplastic transformation by representatives of the 3 major known classes of oncogenic viruses.

7.a.2. Determining whether mammalian cells infected with these oncogenic viruses show enhanced expression of neoplastic transformation after treatment with chemical carcinogens, cocarcinogens or mutagens.

7.b. To determine whether the effects of the chemicals are due to alterations of the host cell DNA.

8. Brief statement of working hypothesis:

Evidence from a variety of sources suggests that cells bearing certain types of genetic lesions are more susceptible than normal cells to additional DNA damage induced by chemicals or viruses, often resulting neoplastic transformation (G.J. Todaro, H. Green and M.R. Swift, Science, 153: 1252, 1966; S.A. Aaronson and G.J. Todaro, Virology, 36: 254, 1968; R.L. O'Brian et al., Intern. J. Cancer, 8: 202, 1971).

Thus, the exposure of cells in culture to carcinogenic chemicals and oncogenic viruses should provide an example for study of the increased carcinogenic risk from multiple exposures to different classes of etiologic agents that are potentially present in the environment. The prior treatment of cell populations with chemical carcinogens or mutagens is expected to result in a marked enhancement of the frequency of neoplastic transformation by subsequent exposure to oncogenic viruses. Similarly, the reversal of the sequence, i.e. the treatment of cells already infected by oncogenic viruses with appropriate chemical agents, especially cocarcinogens, should also yield increased transformation frequencies. Finally, some of these effects can be associated with demonstrable increases in damage to the host cell DNA.

9. Details of experimental design and procedures (append extra pages as necessary)

9.a. Chemicals - Carcinogens of several chemical classes will be used. All agents will be recrystallized or redistilled to ensure their purity. Compounds to be used will be selected from the following list:

9.a.1. Carcinogens (demonstrated as mutagens in various organisms)
7,12-Dimethylbenz(a)anthracene; benzo(a)pyrene; diepoxybutane; bis(chloromethylether); N-acetoxy-2-fluorenyl acetamide; N-methyl-N'-nitro-N-nitrosoguanidine; 4-nitroquinoline-N-oxide; aflatoxin B_1.

9.a.2. Cocarcinogens
Phorbol myristate acetate; anthralin; cigarette smoke condensate.

9.a.3. Mutagens not known to be carcinogens
Ethylmethane sulfonate; nitrogen mustard; ethidium bromide;

9.a.4. Noncarcinogens
Benzo(e)pyrene; 1,2,3,4-diepoxycyclohexane

9.b. Biological Systems

9.b.1. BALB/c-3T3 Cells + SV_{40} Virus

This transformation assay will be carried out essentially as described by Todaro and Green (Proc. Nat. Acad. Sci., 55: 302, 1966; Virology, 28: 756, 1966) and adapted for BALB/c-3T3 cells (S.A. Aaronson and G.J. Todaro, J. Cell. Physiol., 72: 141, 1968). Logarithmically growing cell populations will be exposed to the chemical agents to be tested for intervals up to 48 hours. The concentrations of the chemicals will be selected so that the highest doses result in no more than a 90% reduction in plating efficiency of BALB/c-3T3 cells. Following treatment with chemical agents, the cultures will be washed and incubated for an additional 24 hours in control medium. The cells will be infected with SV_{40} and the population passaged at several cell densities. Transformed

clones will be scored in 10-14 days, and the enhancement of transformation by chemical plus virus treatment over that observed with chemical or virus alone will be determined.

To examine whether chemical agents can influence the outcome of viral transformation, growing cells will be infected with SV_{40}, and these cells will be exposed to chemical agents 24 hours after virus infection. Carcinogens and mutagens will be added to cultures for periods up to 48 hours at concentrations as described above. Co-carcinogens will be used throughout the entire interval of cell growth until the plates are stained for scoring of transformed clones.

Our recent work with early passage rat embryo fibroblasts and alkylating agent carcinogens, such as diepoxybutane, indicates that early chromosomal lesions occur as a result of such treatment (S.R. Wolman, A. Sivak and M.LaRocca, Proc. Am. Assoc. Cancer Res., 14: 38, 1973). Exploratory studies with a few selected chemicals and SV_{40} transformation will be carried out with these rat cells derived from Fisher 344 rat embryos.

Transformed clones from BALB/c-3T3 and Fisher 344 rat cell studies will be picked and tested for oncogenicity by injection of cells subcutaneously into newborn littermates.

9.b.2. BALB/c-3T3 Cells + Mouse Sarcoma Virus (MSV)

The focus assay system described by O'Connor (Perspective in Leukemia, p. 64, 1968, Grune and Stratton, N.Y.) will be employed with BALB/c-3T3 cells and fully competent MSV. The cultures of BALB/c-3T3 cells will be prepared (4×10^5 cells/50 cm dish) in Dulbecco's modification of Eagle's medium with 10 percent fetal calf serum. After 18-20 hours, the plates are drained and virus incubation carried out according to O'Connor. The treatment of cultures with chemicals prior to and after viral infection will be carried out according to the protocol described in 9.b.1. Cultures pretreated with chemical agents for 24 or 48 hours will be adjusted in cell number at the time of passage so that, at the time of virus inoculation, the plates will contain approximately 4×10^5 cells.

Foci of transformed cells will be counted 6-8 days after introduction of virus into the cultures.

clones will be scored in [illegible] and [illegible] of tumor.

Since Freeman and his associates (Proc. Nat. Acad. Sci., 68: 445, 1971; J. Nat. Cancer Inst., 44: 65, 1970) are engaged in the examination of the effects of chemical carcinogens on rat cells chronically infected with Rauscher leukemia virus (Fl15 line), no significant effort with this system is planned. However, it appears useful to test a few selected compounds in both RNA virus systems. Of special interest would be a determination of the effects of tumor promoters on the Fl15 line of rat cells.

9.b.3. Hamster Embryo Cells + Herpes Simplex Virus, Type 2 (HSV).

A procedure for the demonstration of transformation by HSV has been reported by Duff and Rapp (J. Virol., 8: 469, 1971) and will serve as a model for these studies. This system, in contrast to the transformation of BALB/c-3T3 cells by SV_{40} or MSV, requires virus inactivated for its cytopathic effect but retaining oncogenic potential.

Primary hamster embryo cultures from inbred hamsters will be prepared according to standard procedures used in this laboratory (A. Sivak and B.L. Van Duuren, Exptl. Cell. Res., 49: 572, 1968). Secondary cultures will be prepared in Dulbecco's modification of Eagle's medium. The cell layers will be exposed to UV-irradiated HSV, and the cells propagated according to Duff and Rapp, except that plastic Petri dishes will be used throughout.

Treatment of cultures with chemicals will be as described in 9.b.1. except that post-infection treatment with carcinogens may not extend to the 3-4 weeks required for demonstration of transformed clones.

Representive transformed clones will be picked, propogated and tested for malignancy in newborn hamsters.

9.c. Assessment of Genetic Lesions

9.c.1. Cytogenetic Studies

The cell populations employed in this study will be examined for chromosomal alterations after treatment with the various chemicals.

Cell cultures will be harvested 24 or 48 hours after passage or renewal of the medium to maximize the population of dividing cells. After a 3 hour exposure to Velban (0.1 µg/ml), the cells will be harvested by trypsinzation, swelled in 0.38% KCl, fixed in Carnoy's solution and air-dried on slides.

Cells in metaphase from each preparation will be examined after staining with acetoorcein. Whenever possible at least 50 cells per culture will be counted and analyzed. (Slides are scanned at 10X, and cells which are deemed suitable are analyzed under oil immersion (100X) and, if necessary, photographed and karyotyped). Metaphase cells will be scored for chromosome number and for the presence of abnormalities. Gaps*, breaks, deletions and exchange figures will be recorded. Other features affecting the whole of the nuclear material such as pulverization or centromeric splitting will also be recorded. In accordance with the Cytogenetic Standardization Commission of the Environment Mutagen Society, an achromatic area more than a chromatid width will be scored as a break. Results will be tabulated in terms of the number of aberrations per cell in each population and the percentage of aberrant cells per population.. (*Gaps, although recorded will not be included as aberrations).

9.c.2. Biochemical Studies

To analyze for chemically-induced genetic lesions at the molecular level, alkaline sucrose gradient analysis will be performed on H^3-thymidine labeled DNA from treated cells. This procedure is a sensitive assay for single strand breaks in DNA. Cells will be exposed to H^3-thymidine for 2 generations, and the cell density will be adjusted to be identical to the conditions for the chemical + virus studies. After treatment of the labeled cells with chemical at doses and for times used in the transformation assays, the cells will be harvested mechanically (A. Sivak, F. Ray and B.L. Van Duuren, Cancer Res., 29: 624, 1969). Aliquots of washed cell suspensions will be placed on gradients of alkaline sucrose and the DNA sedimented (R.A. McGrath and R.W. Williams, Nature, 212: 534, 1966; J.T. Lett, et al., Nature, 214: 790, 1967).

The gradients will be fractionated by a Buchler Densi-Flow Gradient Sampler, and the radioactivity determined as trichloroacetic acid precipitates collected on Whatman GF-1 glass fiber filters.

This procedure together with the cytogenetic analysis will provide evidence as to whether chemical carcinogens influence viral transformation of mammalian cells because of a direct effect on the genetic material.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The work will be carried out at the A.J. Lanza Laboratories of the Institute of Environmental Medicine, Long Meadow Road, Tuxedo, New York in a laboratory outfitted for tissue culture and biochemical studies. For tissue culture several incubators, sterile box and water-bath are available. Spectrophotometric analyses will be carried out using either Cary or Hitachi UV-visible spectrophotometers; infrared analyses will be performed on Perkin Elmer infrared spectrophotometers. Clinical (International) and high speed (Sorvall SS-1) centrifuges are available for cell fractionation. A Gilson Linear Fractionator with an Ultraviolet Absorption Meter will be used to collect the gradients. There is access to a Model L2-Spinco centrifuge, a Nuclear-Chicago Mark I liquid scintillation spectrometer and a walk-in cold room. Microscopy will be performed using a Nikon Model M inverted microscope with a Sage Instruments, Inc., Model 500 cinephotomicrographic apparatus. Animal facilities exceed the requirements set forth by the Institute of Laboratory Clinical Resources.

11. Additional facilities required:

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

12. Biographical Sketches:-

Name:- Andrew Sivak

Title:- Associate Professor

Birthdate:- REDACTED

Place of Birth:- REDACTED

Major Research Interest:- Mechanisms of Carcinogenesis

Research Experience

Rutgers University, Department of Bacteriology, Research Fellow - In vitro cultivation of Trypanosoma lewisi and effect of antibiotics on T. lewisi in the rat and in vitro. 1956-1957.

Rutgers University, Institute of Microbiology, Research Fellow Tracer and inhibitor studies leading to a partial elucidation of the route of biosynthesis of actinomycin and studies of the pathways of tryptophan metabolism in Streptomyces antibioticus. 1957-1960

Organisch-Chemisches Institute, University of Vienna, Austria. Post-Doctoral Fellow - Studied pathway of inositol catabolism in Schwanniomyces occidentails and the inducible enzyme which was responsible for the first oxidative step. 1960-1961

A.D. Little, Inc., Cambridge, Mass., Senior Biochemist - Devised analytical methods for the estimation of experimental cancer chemotherapeutic agents. Physiological distribution and toxicity studies of these agents in mice, rats, dogs, and monkeys. Mode of anti-tumor activity studies in mice bearing ascitic leukemia and in in vitro systems derived from these neoplasms. 1961-1963

Biodynamics, Inc., East Millstone, N.J., Director of Research for a consulting laboratory in the biological sciences. Scientific direction of biological and biochemical work dealing with studies in microbiology, physiology, biochemistry and toxicology. 1963-1964

N.Y.U. Medical Center, Institute of Environmental Medicine. Associate Research Scientist, 1964-1968; Assistant Professor, 1968-1971; Associate Professor, 1971 - Study of cell membrane and control of cell division; mechanisms of tumor promotion in cell culture and animal systems; tumor promoting properties of cigarette tar; neoplastic transformation in cell culture systems; cytogenetic studies with chemical carcinogens and chromosome structure.

Other Experience

1952 - 1955	U.S. Navy, Commissioned Officer.
1959	Oak Ridge Institute of Nuclear Studies - Isotopes techniques training.

Education

B.S.	Rutgers Univ., New Brunswick, N.J.	1952	Biology
M.S.	Rutgers Univ., New Brunswick, N.J.	1957	Bacteriology
Ph.D.	Rutgers Univ., New Brunswick, N.J.	1960	Microbiol. - Biochem.
U.S.P.H.S.	Post-Doct. Fellow Organisch-Chemisches Inst., Univ. of Vienna, Austria	1960-1961	

Professional Societies

REDACTED

R.I.C. Medical Center, Institute of Environmental Medicine
Associate Research Scientist, 1966-1969; Assistant Professor, 1969-1971; Associate Professor, 1971 - Study of cell membrane [illegible]

13. Publications

A. Sivak and B.L. Van Duuren, Phenotypic expression of transformation: Induction in cell culture by a phorbol ester. Science, 157: 1443-1444 (1967).

A. Sivak and B.L. Van Duuren, A cell culture system for the assessment of tumor-promoting activity. J. Nat. Cancer Inst., 44: 1091-1097 (1970).

A. Sivak and B.L. Van Duuren, Cellular interactions of phorbol myristate acetate in tumor promotion. Chem.-Biol. Interactions, 3: 401-411 (1971).

A. Sivak and S.R. Wolman, Classification of cell types: Agglutination and chromosomal properties. In Vitro, 8: 1-6 (1972).

A. Sivak, Induction of cell division. Role of cell membrane sites. J. Cell. Physiol., 80: 167-174 (1972).

American Association for Cancer Research

Environmental Mutagen Society

4.

14. First year budget:

A. Salaries (give names or state "to be recruited"). Professional (give % time of investigator(s) even if no salary requested).	% time	Amount
Andrew Sivak, Principal Investigator	R	-
Cell Biologist-Virology (Assoc. Res. Scientist) to be recruited	R	-
Technical		
Susan Kulina (Research Technican)		REDACTED
Animal Caretaker		
Fringe Benefits (14% S & W)		REDACTED
	Sub-Total for A	$ 31,037.

B. Consumable supplies (by major categories)

Glassware, Tissue Culture Vessels	$ 2,500.
Medium components, serum, biologicals	2,500.
Chemicals, Isotope labeled biochemicals	2,000.
Liquid nitrogen, carbon dioxide	1,200.
Mice, rats hamsters, animal food and bedding	2,000.
Sub-Total for B	$ 10,200.

C. Other expenses (itemize)

Domestic travel- scientific meetings	$ 600.
Foreign travel - International Cancer Congress Florence, Italy, October, 1974.	700.
MS preparation	500.
Books and Reprints	300.
Sub-Total for C	$ 2,100.
Running Total of A + B + C	$ 43,337.

D. Permanent equipment (itemize)

Biohazard Hood	$ 3,000.
Tissue Culture Incubator	2,000.
Sub-Total for D	$ 5,000.
E	6,501.
Total request	$ 54,838.

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$33,657.	$11,000.	$2,000.	-	$6,999.	$53,656.
Year 3	$36,181.	$12,000.	$2,000.	-	$7,527.	$57,708.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Principal Investigator			
Neoplastic Growth and Cell Cycle Control	NIH - CA-12503	$25,048.	5/1/73 to 4/30/74
Co-Investigator			
Initiation and Promotion in Carcinogenesis	NIH - CA-08580	38,396.	1/1/73 to 12/31/73
Tobacco Carcinogenesis	NIH - N01-CP-3-3241	84,620.	12/29/72 to 12/28/73
Carcinogen Induced Chromosome Changes and Transformation	NIH - CA-13821	23,871.	9/1/72 to 8/31/73

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Neoplastic Growth and Cell Cycle Control	NIH - CA-12503	Renewal	5/1/74 - 4/30/77

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy, Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Andrew Sivak

Signature Andrew Sivak Date 7/25/73

Telephone 212-679-3200 (Area Code) 5-228 (Number Extension)

Checks payable to

New York University Medical Center

Mailing address for checks

550 First Ave., New York, N.Y. 10016

Atten. John Ballow, V.P. For Finance

Responsible officer of institution

Typed Name Peter Arakelian

Title Vice President for Facilities and Services

Signature [illegible] Date 7/26/73

Telephone 212-679-3200 (Area Code) 2033 (Number Extension)

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